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Residue Study of Ivermectin in Plasma, Milk, and Mozzarella Cheese Following Subcutaneous Administration to Buffalo (*Bubalus bubalis*)

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The distribution of ivermectin in buffalo plasma and milk after administration of a single subcutaneous dose (0.2 mg kg^{-1} b.w.) was studied. Ivermectin reached the maximal concentration in plasma (28.5 ± 1.7 ng mL^{-1}) and milk (23.6 ± 2.6 ng mL^{-1}) after 2.4 ± 0.32 and 2.8 ± 0.44 days, respectively. The drug showed a parallel disposition in milk and plasma, with a ratio of 1.12 ± 0.16. Ivermectin concentrations were detected in mozzarella cheese obtained from milk collected on days 1, 3, 4, and 20 following administration. The highest values (81.4 ± 3.26 ng g^{-1}) were found in the cheese produced on day 3 and were 4-fold higher than those present in the milk.

KEYWORDS: **Ivermectin; buffalo; plasma; milk; mozzarella cheese**

INTRODUCTION

Ivermectin is a macrocyclic disaccharide anthelmintic agent which belongs to the chemical family of the avermectins and consists of a mixture containing at least 80% 22−23 dihydroavermectin B_{1a} and less than 20% 22−23 dihydroavermectin B_{1b} (*1*). The compound is used against adult and larval forms of different nematodes species and ectoparasites of food-producing animals and is included in Annex I of the Council Regulation (EEC) 2377/90 with MRLs ranging from 15 to 100 μg kg^{-1} depending on the species. The maximum levels of the marker residue H_2B_{1a} are 15 μg kg^{-1} for the fat and 20 μg kg^{-1} for the liver from pigs, sheep, and horse; 40 μg kg^{-1} for the fat and 100 μg kg^{-1} for the liver from cattle; and 20 μg kg^{-1} for the kidney and muscle, 50 μg kg^{-1} for the liver, and 100 μg kg^{-1} for the fat from deer and reindeer species (*2*).

The pharmacokinetic behavior of ivermectin depends on the route of administration, the formulation, and the animal species. The high lipophilicity of this drug accounts for its long residence in plasma and excretion with milk.

Detailed ivermectin pharmacokinetic studies have been conducted for horse (*3*), pig (*4*), cattle (*5*), sheep (*6*), and goat (*7*). Even if its efficacy against cutaneous parasites and gastrointestinal nematodes of buffalo is proved (*8*), information concerning pharmacokinetics are lacking for this species.

Italy, with 200,000 buffaloes, has the most buffalo breedings of any country in the European Community; 90% of these buffalo are located in central and southern Italy. The production of buffalo milk accounts for only 1% of overall Italian milk production (82,000 t in 2000), but it is almost entirely used to produce "mozzarella di bufala campana" cheese, which is the third Protected Designation of Origin (PDO) Italian cheese. PDO is the term used to describe foodstuffs that are produced and prepared in a given geographical region using recognized processes.

Although ivermectin is not approved for use in lactating animals the possibility of an unapproved use should be considered. As ivermectin residues persist for long periods in milk from cow (*9*) and goat (*7*) the aim of this study was to investigate the ivermectin pharmacokinetic in buffalo plasma and milk and to evaluate to what extent ivermectin levels may be present in mozzarella cheese following a subcutaneous administration to the lactating animals.

MATERIALS AND METHODS

Apparatus. An automated SPE system, Aspec XL Gilson (Worthington, OH), was used for the SPE cleanup. The SPE columns were C18 cartridges ISOLUTE, 500 mg (IST Ltd, U.K.).

The HPLC analyses were performed on a Merck-Hitachi Lachrom instrument (Merck, Darmstadt, Germany) equipped with a pump (L-7100), an interface (D-7000), a fluorescence detector (L-7480), and an

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autosampler (L-7250). The HPLC column was a Lichrospher 100 RP-18, 250 × 4 mm i.d., with a packing of 5-μm particles (Merck).

Chemicals and Reagents. Methanol and acetonitrile (HPLC grade), chloroform, and tertbutylmethyl ether were from Carlo Erba (Milano, Italy). Water was obtained from a Milli-Q plus ultrapure water system (Millipore, Bedford, MA). Triethylamine, 1-methylimidazole, acetic anhydride, and *N,N*-dimethylformamide were from Sigma Chemical Co (St. Louis, MO).

Standard Solutions. Ivermectin standard was obtained from Sigma. The standard stock solution of ivermectin was prepared in methanol at 100 mg L^{-1} and stored at −20 °C. A working standard solution at 0.25 μg L^{-1} was obtained by diluting stock solution with methanol on the day of use.

Animal Experiments. Five lactating buffaloes (*Bubalus bubalis*) (400−500 kg bw), producing 9.4 ± 2.07 kg of milk daily and maintained indoors during the whole experimental period, were used in this trial. Ivermectin was given in a single subcutaneous administration of the commercially available formulation used for cattle, i.e., 0.2 mg kg^{-1} (Ivomec 1% Msd Agvet, Rahway, NJ).

Sample of blood and milk were collected from each animal prior to treatment, to obtain "blank" tissues, and subsequently on days 0.125, 0.25, 0.5, 1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8, 11, 14, 17, 20, 23, 26, and 30. Blood samples were centrifuged at 3000 g for 20 min and the recovered plasma was transferred to plastic vials. Milk aliquots were collected in glass bottles and transferred to 5-mL tubes. The milk collected on days 1, 3, 4, and 20 was used to produce mozzarella cheese according to the traditional procedures established by the DPCM 10.05., 1993 (*10*). Cheese samples were placed in labeled plastic bags.

Blood, milk, and cheese samples were rapidly transported on dry ice to the laboratory and kept frozen at −20 °C until analyzed.

Sample Preparation. Plasma samples were extracted according to De Montigny et al. (*11*). One mL of acetonitrile was added to 1 mL of plasma. After these were mixed for 20 min, the solutions were centrifuged at 2000*g* for 2 min, and the supernatants were applied to a C18 SPE cartridge preactivated with methanol (2 mL) and water (2 mL). The column was washed with 2 mL of water/methanol(75:25, v/v) and ivermectin was eluted with 1 mL of methanol. This eluate was evaporated to dryness under a gentle stream of nitrogen.

Milk and mozzarella cheese samples were extracted using the method described by Dusi et al. (*12*). Acetonitrile (5 mL) was added to 5 mL of milk or to 5 g of cheese in a 20-mL glass centrifuge tube, and the mixture was shaken with a horizontal shaker at high speed for 10 min. The tube was then put into an ultrasonic bath for 10 min. After the shaken mixture was centrifuged for 5 min at 3000*g*, the supernatant layer was poured from the tube, through a folded filter paper, into a 50-mL flat-bottomed flask. The sample was re-extracted as previously described with 5 mL of acetonitrile. The acetonitrile portions were combined and the filter was washed with 3 mL of acetonitrile. Water (20 mL) and triethylamine (30 μL) were then added to the flask.

The solution was mixed and then applied to a C18 SPE cartridge (500 mg/6 mL), pretreated with, in sequence, 3 × 5 mL of methanol, 5 mL of acetonitrile, and 3 × 5 mL of water/acetonitrile mixture (70:30, v/v) containing 0.1% triethylamine. The column was washed with 2 × 5 mL of water/acetonitrile (50:50, v/v), and ivermectin was eluted with 7 mL of terbutylmethyl ether. The tube was stored overnight at −20 °C. The cool, clear supernatant organic layer was evaporated to dryness.

The residue was derivatized in a tube with 100 μL of 1-methylimidazole/acetic anhydride/dimethylformamide (2:6:9, v/v) at 95 °C for 60 min. The tube was cooled and 1 mL of chloroform was added. This solution was vortex-mixed, and then the dark brown solution was transferred to a silica SPE cartridge (500 mg/6 mL) previously activated with 3 mL of chloroform. The tube was rinsed with 9 mL of chloroform. The eluate was evaporated to dryness under nitrogen. The residue was dissolved in 200 μLof methanol, and 25 μL was injected into the chromatographic system.

HPLC−FL Analysis. Ivermectin was analyzed in isocratic elution at 1.2 mL/min, at room temperature, with a mobile phase consisting of methanol/water (98:2, v/v). Excitation and emission wavelengths were 364 and 470 nm, respectively.

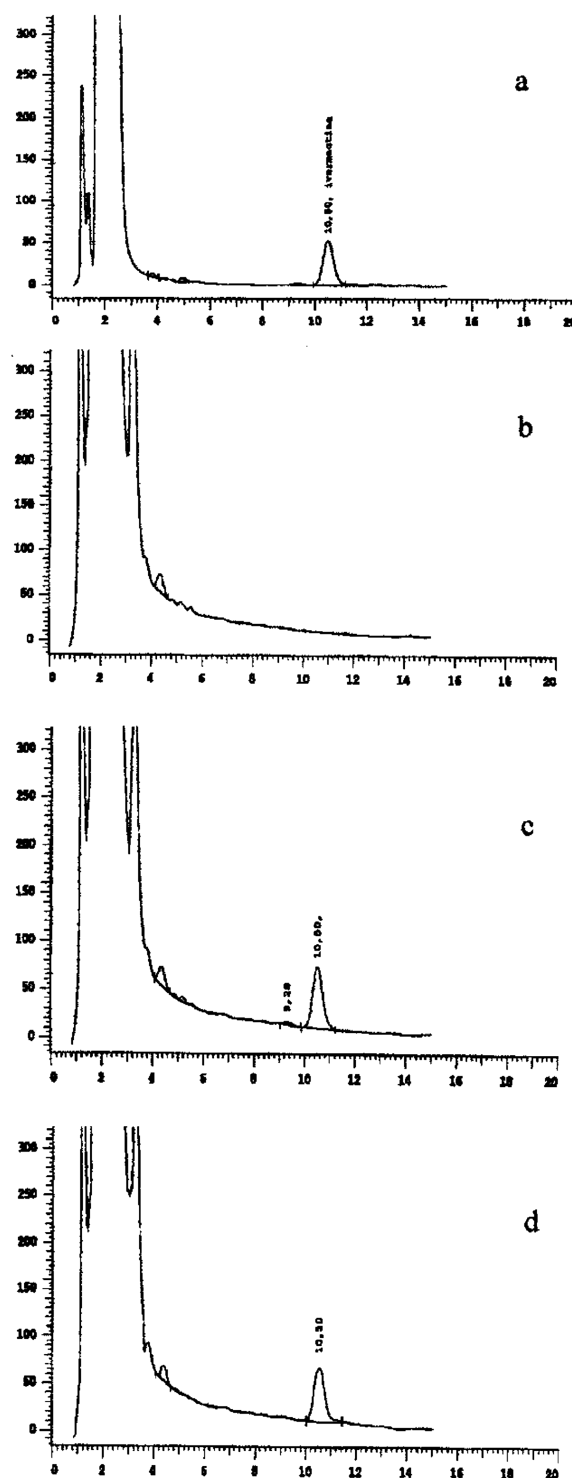

Figure 1. Chromatograms of (a) 20 ng mL^{-1} ivermectin standard, (b) control mozzarella cheese, (c) mozzarella cheese fortified with 40 ng mL^{-1}, and (d) mozzarella cheese produced with milk from inoculated buffaloes.

Method Calibration. The methods reported by De Montigny et al. (*11*) and Dusi et al. (*12*) for ivermectin determination in plasma and milk were used, respectively. The method proposed for milk samples was followed, with minor changes, to determine the residues in cheese.

The linearity of the methods was determined with the HPLC analysis of different aliquots from the working solution at 0.25 μg L^{-1}, corresponding to 2.5, 5, 10, 20, and 40 μg kg^{-1} (tissue equivalency). Calibration curve of ivermectin was constructed by plotting peak area (*y*) vs standard concentration in μg kg^{-1} (*x*).

To determine accuracy, aliquots of the drug-free samples of milk, plasma, and cheese were spiked with ivermectin at four levels: 5, 10,

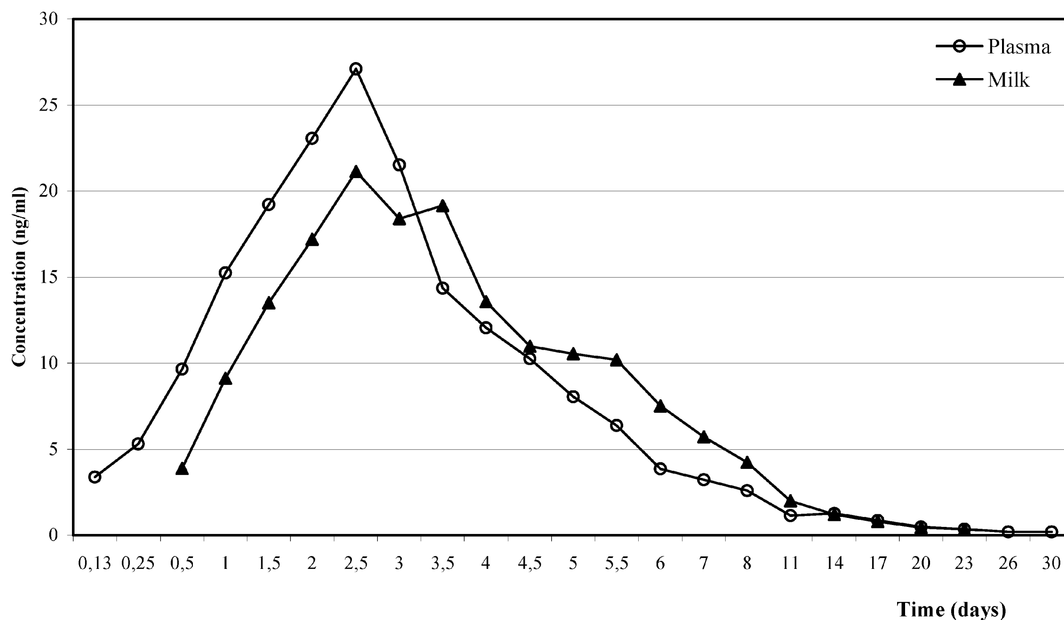

Figure 2. Concentration–time profile of ivermectin (mean values) in buffalo plasma (○) and milk (▲) following a single sc administration of 0.2 mg kg^{-1}.

20, and 40 μg kg^{-1} (**Figure 1**). Three replicates were prepared at each concentration level. Fortified samples were extracted, cleaned-up, and determined after derivatization as described earlier.

The detection limit (LOD), calculated from noise signal-to-noise of 20 blank samples, was 0.2 ppb. The limit of quantification (LOQ) calculated according to council directive 96/23/EC criteria (*13*) in plasma, milk, and cheese was 5 ng/g. The precision of the methods, expressed as within-day repeatability, was determined by analyzing samples spiked with ivermectin standard solution. The specificity of the methods was confirmed by analysis of blank samples. No interferent peak eluted at the same retention time of ivermectin.

Pharmacokinetic Analysis. Plasma and milk concentration vs time curves obtained after each treatment in each individual animal were fitted with the TopFit software (*14*). Pharmacokinetic parameters were determined using a noncompartmental model. The peak concentration (C max) and the time to peak concentration (T max) were read from the plotter concentration−time curve in each individual animal. The area under the concentration−time curves (AUC) was calculated by the trapezoidal rule (*15*) and further extrapolated to infinity by dividing the last experimental concentration by terminal slope (β).

Statistical moment theory was applied to calculate the mean plasma residence time (MRT) for ivermectin, as follows:

$$\mathrm{MRT} = \frac{\mathrm{AUMC}}{\mathrm{AUC}}$$

where AUMC is the area under the curve of the product of time and drug concentration vs time from 0 to infinity (*16*).

The phamacokinetic parameters are reported as mean ± SD with $n = 5$ and were statistically compared using the analysis of variance (ANOVA) of SAS (*17*). Values were considered significantly different at $P < 0.05$.

RESULTS AND DISCUSSION

The analytical method used to extract, derivatize, and quantify the plasma, milk, and cheese ivermectin concentrations by chromatographic analysis using the fluorescence detector was shown to be adequate. In fact the regression analysis of the data obtained from the standard solutions showed that the ivermectin responses were linear over the range of the concentrations examined (2.5−40 μg kg^{-1}). The correlation coefficient (r^2) obtained exceeded 0.99, demonstrating a good linearity. The overall ivermectin recoveries were 77.8, 82.5, and 80.9% for plasma, milk and cheese, respectively. Intra-assay variations ranged between 10.0% (milk) and 13.4% (plasma).

Table 1. Pharmacokinetic Parameters for Ivermectin in Plasma Obtained after Subcutaneous Administration to Buffalo at 0.2 mg kg^{-1}

	animal					
parameter[a]	1	2	3	4	5	mean ± SD
C_{max} (ng mL^{-1})	28.2	29.1	28.2	26.0	31.0	28.5 ± 1.76
T_{max} (h)	72.0	58.0	58.2	57.8	48.0	58.8 ± 7.63
AUC (ng d mL^{-1})	118.3	106.6	84.6	87.8	96.8	98.8 ± 2.20
$t_{1/2\,el}$ (h)	44.5	63.3	49.7	37.2	36.8	46.3 ± 6.71
MRT (h)	83.4	97.7	68.1	78.8	74.7	80.5 ± 9.12

[a] C_{max}, peak plasma concentration; T_{max}, time to reach peak concentration; AUC,: area under the concentration–time curve; $t_{1/2\,el}$: elimination half-life; MRT: mean residence time.

Table 2. Pharmacokinetic Parameters for Ivermectin in Milk Obtained after Subcutaneous Administration to Buffalo at 0.2 mg kg^{-1}

	animal					
parameter[a]	1	2	3	4	5	mean ± SD
C_{max} (ng mL^{-1})	22.4	27.6	23.6	22.5	21.9	23.6 ± 2.6
T_{max} (h)	58.2	58.3	82.5	72.0	72.1	68.6 ± 10.6
AUC (ng d mL^{-1})	150.1	99.5	94.4	94.6	88.5	105.4 ± 25.5
$t_{1/2\,el}$ (h)	85.1	53.8	60.1	52.8	56.5	61.6 ± 13.3
MRT (h)	122.8	105.1	101.4	108.2	110.4	109.5 ± 8.3

[a] C_{max}, peak plasma concentration; T_{max}, time to reach peak concentration; AUC, area under the concentration–time curve; $t_{1/2\,el}$, elimination half-life; MRT, mean residence time.

The use of an automated system in the cleanup procedure made the methods reliable and faster, reducing the tedious and time-consuming phase of the sample purification (*18*).

The pharmacokinetic parameters are reported in **Tables 1** and **2** and in **Figure 2**.

After a single sc administration of 0.2 mg kg^{-1}, the plasma ivermectin maximal concentration was 28.5 ± 1.76 ng mL^{-1} after 2.4 ± 0.32 d (**Table 1**; **Figure 2**). This level is lower than that obtained in cow (42.8 ng mL^{-1}) by Bogan and Mc Kellar (*6*), but higher than that determined in goat (6.12 ng mL^{-1}) reported by Alvinerie et al. (*7*) after administration of the same sc ivermectin dose.

The different concentrations are correlated to different AUC values (98.8 ng/day/mL^{-1} for buffalo; 459 ng/day/mL^{-1} for cow (*6*); and 46 ng/day/mL^{-1} for goat (*7*)). The buffalo values indicate low levels of parenteral drug in plasma and suggest a buffalo ivermectin bioavailability lower than that of cow but higher than that of goat. McKellar and Benchaoui (*19*) suppose that the low plasma concentrations observed in buffalo could be one of the reasons for the emergence of resistant nematodes. Bogan and McKellar (*6*) relate the above-mentioned differences in ivermectin distribution to several factors, such as the size of the body compartments and the amount of subcutaneous fat.

The ivermectin mean residence time (MRT) in buffalo plasma (3.3 ± 0.3 d) was not different from those observed in cow and goat. The milk ivermectin levels reached the maximal concentration of 23.6 ± 2.60 ng mL^{-1} after 2.8 ± 0.44 days (**Table 2**; **Figure 2**). Plasma and milk residues decreased gradually and were below the detection limit after 28 ± 2.34 days. The milk/plasma ivermectin ratio, determined by using the area under the curve (AUC) values, was 1.12 ± 0.16. The elimination half-life was slightly higher for milk than for plasma (2.56 d vs 1.93 d).

The ivermectin concentrations in buffalo milk were found to be rather similar to that in the plasma levels (**Figure 1**), in accordance with previous studies concerning cows, ewes, and goats, (*6*, *7, 9*). The amount of the drug recovered from milk during the whole experimental period was 0.94% $\pm$ 0.23% of the administered dose. This percentage is more similar to that recovered from goat (0.31%) (*7*) than that obtained from cow (5.46%) (*9*) and sheep (4%) (*20*).

The mean levels of ivermectin residues in the mozzarella cheeses produced with milk collected on days 1, 3, 4, and 20 following administration were 69.6 ± 2.18 ng g^{-1}, 81.4 ± 3.26 ng g^{-1}, 42.2 ± 2.45 ng g^{-1}, and 3.8 ± 1.89, ng g^{-1}, respectively.

Ivermectin inhibits the parasite motility by acting as an agonist of γ-amino-butyric-acid (GABA), the neurotransmitter substance mediating transmission of inhibitory signals, which is present also in mammal central nervous systems. The drug is a macrolide of high molecular weight which does not cross the adult blood−brain barrier of mammals, but its permeability is known to be affected by several factors (*1*). Being a lipid-soluble substance, ivermectin is well absorbed from humans after oral administration, and for this reason no MRLs have been set yet for milk in EU. Recently, the Committee for Veterinary Medicinal Products recommended that the Codex Alimentarius draft MRL for bovine milk at 10 μg/kg should not be supported because no information is available to calculate the ivermectin residue intake from milk, and there is a concern that the addition of a MRL for milk should result in a daily residue intake, which may exceed the Acceptable Daily Intake (ADI) (*21*).

Though the administration of ivermectin is prohibited for lactating animals producing milk for human consumption, the consequences of an illegal use could always be considered.

The ivermectin concentrations found in milk from buffaloes which had received 0.2 mg kg^{-1} in a single subcutaneous dose do not arouse particular concern. Human consumption of liquid buffalo milk is very limited in western countries, as the milk is used almost entirely to produce milk products. It is worth noting, however, that Toutain et al. (*9*) subjected milk samples to a housefly bioassay and found that the mortality rate of groups that had received milk containing more than 20 ppb was significantly higher than that of the controls.

The values observed in mozzarella cheese, however, deserve careful attention. The cheese manufactured with milk collected from the inoculated buffaloes on day 3 after administration revealed a concentration of ivermectin 4-fold higher than that in the milk. On day 20, when the drug milk residues were <0.6 ng g^{-1}, the levels in cheese were 3.8 ng g^{-1}.

Further studies are prompted to evaluate hazards arising from ingestion of different dairy products. Training and monitoring programs for farmers to verify compliance with the regulation in effect are suggested as well.

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